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Cis-Acting Functions of Alfalfa Mosaic Virus Proteins Involved in Replication and Encapsidation of Viral RNA

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cDNA clones of RNAs 1 and 2 of alfalfa mosaic virus (AMV) were slightly modified to permit transcription of infectious RNAs with T7 RNA polymerase. Together with transcripts of an available clone of AMV RNA 3, these transcripts were used to study *cis*- and *trans*-acting functions of AMV proteins in protoplasts from nontransgenic tobacco plants and from plants transformed with the P1 and P2 genes, encoded by RNAs 1 and 2, respectively. Transgenic P1 was unable to complement mutations in the P1 gene in RNA 1, pointing to a *cis*-acting function of P1 in RNA 1 replication. A study of the replication of RNA 3 mutants in nontransgenic protoplasts revealed that coat protein (CP) expressed from RNA 3 in the inoculum is required in *trans* for replication and encapsidation of RNAs 1 and 2 but is required in *cis* for replication and encapsidation of RNA 3. CP is required in the inoculum to initiate infection of nontransgenic plants and protoplasts. When protoplasts expressing both P1 and P2 (P12 protoplasts) were infected with RNAs 1, 2, and 3, initiation of replication of RNAs 1 and 2 required the presence of CP in the inoculum, whereas the initiation of replication of RNA 3 did not. This demonstrated that CP expressed from RNA 3 cannot substitute for the early function of CP in the inoculum. The results showed that CP in the inoculum is required to permit viral minus-strand RNA synthesis, whereas CP expressed from RNA 3 after the initiation of infection is required for plus-strand RNA synthesis.

INTRODUCTION

Complementation studies have revealed *cis*-acting functions for protein coding sequences in the RNA genomes of many plant and animal viruses. Such *cis*-acting sequences may encompass large regions of genes encoding proteins involved in replication or encapsidation of viral RNA. It has been speculated that some of these sequences could reflect a coupling between translation and replication of viral RNA or a *cis*-preferential function of the encoded protein in virus replication. Viruses for which *cis*-acting coding sequences have been reported include poliovirus (Novak and Kirkegaard, 1994), mouse hepatitis virus (de Groot *et al.,* 1992), clover yellow mosaic virus (White *et al.,* 1992), cowpea mosaic virus (van Bokhoven *et al.,* 1993), turnip yellow mosaic virus (Weiland and Dreher, 1993), barley stripe mosaic virus (Zhou and Jackson, 1996), tomato bushy stunt virus (Scholthof and Jackson, 1997), and tobacco etch virus (Mahajan *et al.,* 1996; Schaad *et al.,* 1996). We have analyzed *cis*-acting coding sequences in the tripartite genome of alfalfa mosaic virus (AMV). RNAs 1 and 2 of AMV encode the proteins P1 and P2, respectively, which are associated with purified AMV replicase preparations (Quadt *et al.,* 1991). P1 contains an N-terminal methyltransferase motif and a C-terminal helicase motif, whereas P2 contains a polymerase motif. RNA 3 encodes the movement protein P3 and the coat protein (CP), which is translated from the subgenomic RNA 4. A unique feature of AMV and ilarviruses is the phenomenon that a mixture of RNAs 1, 2, and 3 is not infectious to plants or protoplasts unless a few molecules of CP or RNA 4 are added to the inoculum. This early function of CP has been termed "genome activation" (reviewed in Jaspars, 1985). An infectious clone of AMV RNA 3 has been obtained that can be transcribed into RNA that replicates in transgenic tobacco plants expressing the P1 and P2 proteins (P12 plants; Neeleman *et al.,* 1991; Taschner *et al.,* 1991). In contrast to nontransgenic plants, no CP is required in the inoculum for infection of P12 plants with RNA 3. This system has been used for extensive investigations on *cis*-acting sequences involved in replication of RNA 3. Deletions or frame-shifts in the P3 gene did not affect RNA 3 accumulation in P12 protoplasts, but similar mutations in the CP gene interfered with plus-strand RNA accumulation (van der Kuyl *et al.,* 1991a). When P12 protoplasts were coinoculated with a P3 mutant and a CP mutant, the wild-type (WT) CP expressed by the P3 mutant did not enhance the low level accumulation of the CP mutant (van der Vossen *et al.,* 1996). Although this indicated that CP is required in *cis* for RNA 3 accumulation, the possibility could not be fully ruled out that the two types of RNA 3 mutants infected nonoverlapping populations of the P12 protoplast preparation.

Cis-acting functions in AMV RNAs 1 and 2 have been

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studied using infectious DNA copies of these RNAs flanked by the CaMV 35S promoter and *nos* terminator. Infection with 35S-cDNAs 1, 2, and 3 of tobacco plants transformed with the P1 gene (P1 plants) resulted in replication of all three genomic RNAs, but mutations in the P1 gene in the 35S-cDNA 1 construct interfered with RNA 1 accumulation without affecting accumulation of RNAs 2 and 3. Similarly, mutations that caused an amino acid change in the GDD motif in P2, but not silent mutations in this motif, interfered with coreplication of RNA 2 with RNAs 1 and 3 in transgenic P2 plants (van Rossum *et al.,* 1996). This indicated that P1 and P2 were required in *cis* for the accumulation in plants of RNAs 1 and 2, respectively. Because we were unable to infect protoplasts with the 35S-cDNAs, no distinction could be made between a *cis* requirement in RNA replication or cell-to-cell movement of the virus.

Here we report the construction of cDNA clones of AMV RNAs 1 and 2 that can be transcribed *in vitro* into infectious RNAs with T7 RNA polymerase. These transcripts were used to reinvestigate *cis*-acting functions of P1, P2, and CP in P1, P2, P12, and nontransgenic protoplasts. P1 was found to be required in *cis* for RNA 1 replication rather than for cell-to-cell movement. The hypothesis that CP is required in *trans* for the synthesis of plus-strand RNAs 1 and 2 was confirmed, and it was shown that CP is required in *cis* for both replication and encapsidation of RNA 3. Initiation of replication of RNAs 1 and 2 in P12 protoplasts was found to be dependent on CP in the inoculum, in contrast to initiation of RNA 3 replication. This demonstrates that CP expressed from RNA 3 is unable to fulfill the early function of CP in the inoculum. The results indicate that CP in the inoculum and CP expressed from RNA 3 after the initiation of infection function in different steps of viral RNA replication.

RESULTS

Infectivity of AMV transcripts

When full-length DNA copies of AMV RNAs 1 and 2 were precisely fused to the T7 promoter, no transcription of the constructs by T7 RNA polymerase was observed *in vitro*. Insertion of two G residues between the T7 promoter and the viral cDNAs resulted in efficient transcription by T7 RNA polymerase, but no infectivity was detectable when the RNA 1 and 2 transcripts were inoculated on plants together with virus particles isolated from P12 tobacco plants that had been infected with RNA 3 transcripts. When one G residue was inserted between the T7 promoter and viral cDNA, low amounts of RNA 1 and 2 transcripts were obtained with an infectivity just above the detection level but not of practical use (results not shown). Apparently, the 5′ sequence GUUUUU of RNAs 1 and 2 was too poor in purine residues to permit efficient T7 promoter activity. On the other hand, the 5′ sequence GUAUUA of RNA 3 permitted efficient transcription of the cDNA 3 clone pAL3 by T7 RNA polymerase into transcripts without 5′ nonviral nucleotides and with an infectivity resembling that of native RNA 3 (Neeleman *et al.,* 1991). To solve the problems with transcription of cDNAs 1 and 2, the 5′ sequences of these cDNAs were changed into GTATTT or GTATTA. Both modifications permitted efficient transcription by T7 RNA polymerase of cDNAs 1 and 2 into transcripts without 5′ nonviral nucleotides that were highly infectious (van Rossum, 1998). Here we used clones pUT17A and pUT27A, which are transcribed by T7 RNA polymerase into RNA 1 and 2 molecules, respectively, with a 5′ sequence changed from GUUUUU into GUAUUU. Transcripts of cDNAs 1 and 2 were biologically active only when they were capped *in vitro* (result not shown); transcripts of cDNA 3 were biologically active regardless of whether they were capped *in vitro* (Neeleman *et al.,* 1991).

Cis-acting functions of P1 and P2

Transgenic P1 plants support replication of AMV RNAs 2 and 3 without the need for RNA 1 in the inoculum, and P2 plants support replication of RNAs 1 and 3 in the absence of RNA 2. However, after inoculation with a mixture of RNAs 1, 2, and 3, RNAs 1 and 2 coreplicate with the other RNAs in P1 and P2 plants, respectively (Taschner *et al.,* 1991). Using 35S-cDNA clones, it was shown that mutations in the P1 or P2 gene that affected the infection of nontransgenic plants also abolished the ability of RNAs 1 and 2 to coreplicate with other AMV RNAs in P1 and P2 plants (van Rossum *et al.,* 1996). Here, we used T7 RNA polymerase transcripts of clones pUT17A and pUT27A to analyze *cis*-acting functions of P1 and P2 in protoplasts. Figure 1A (lane 2) shows the accumulation of plus-strand RNAs 1–4 in P1 protoplasts infected with native genomic RNAs and CP isolated from virions. Lane 3 (Fig. 1A) shows the infection of P1 protoplasts with a mixture of CP and RNAs 1–3 transcribed from pUT17A, pUT27A, and pAL3, respectively. In Fig. 1C, the blot of Fig. 1A was hybridized with a probe detecting plus-strand RNA 1 only. In nontransgenic protoplasts, identical patterns of RNA accumulation were observed after inoculation with native RNAs or T7 transcripts (result not shown), but in P1 protoplasts inoculated with transcripts, the accumulation of RNA 1 was slightly reduced compared with the WT infection (Figs. 1A and 1C, lanes 2 and 3). Frame shifts near the 5′ end of the P1 gene (mutant 1-fsP) or near the 3′ end of the P1 gene (mutant 1-fsN) in clone pUT17A abolished the ability of the RNA 1 transcript to coreplicate with RNAs 2 and 3 in the P1 protoplasts (Fig. 1A and 1C, lanes 4 and 5). This indicates that the inability of these mutants to accumulate in P1 plants (van Rossum *et al.,* 1996) was due to a defect in RNA replication rather than a defect in cell-to-cell spread.

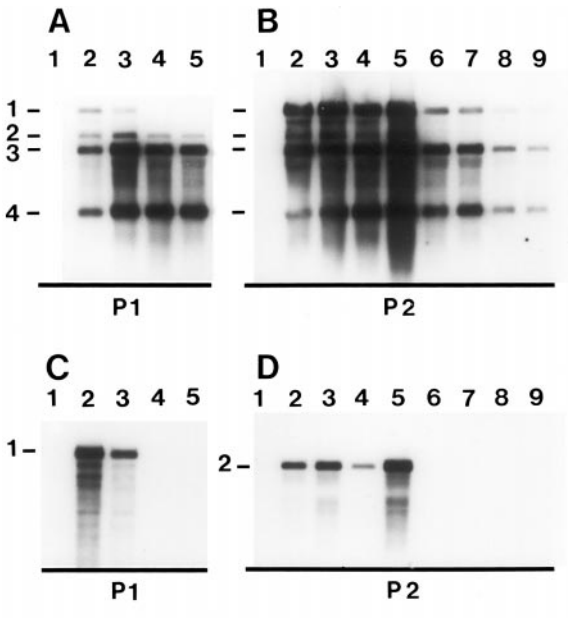

FIG. 1. Replication of *in vitro* transcribed AMV RNAs in P1 and P2 protoplasts. P1 protoplasts (A and C) were mock inoculated (lanes 1) or inoculated with WT RNAs 1–4, extracted from AMV virions (lanes 2); T7 transcripts of clones pUT17A, pUT27A, pAL3, and CP (lanes 3); T7 transcripts of clones 1-fsP, pUT27A, pAL3, and CP (lanes 4); and T7 transcripts of clones 1-fsN, pUT27A, pAL3, and CP (lanes 5). P2 protoplasts (B and D) were mock inoculated (lanes 1) or inoculated with various AMV RNA preparations (lanes 2–9). In lanes 2–5 of B and D, the protoplasts were inoculated with RNAs 1–4 extracted from virions that had been isolated from tobacco plants infected with 35S-cDNAs 1–4 (lanes 2); 35S-cDNAs 1; 2-GDD-S; 3 and 4 (lanes 3); T7 transcripts of clones pUT17A, pUT27A, pAL3, and CP (lanes 4); and T7 transcripts of clones pUT17A, 2-GDD-S, pAL3, and CP (lanes 5). In lanes of 6–9 of B and D, the protoplasts were directly inoculated with T7 transcripts of clones pUT17A, pUT27A, pAL3, and CP (lanes 6); pUT17A, 2-GDD-S, pAL3, and CP (lanes 7); pUT17A, 2-GGD, pAL3, and CP (lanes 8); and pUT17A, 2-VDD, pAL3, and CP (lanes 9). RNA extracted from the protoplasts was analyzed by Northern blot hybridization. In A and B, the probes detected plus-strand RNAs 1–4; in C and D, the probes detected plus-strand RNA 1 and RNA 2, respectively. The position of RNAs 1–4 is indicated in the left margin.

Figures 1B and 1D show the infection of P2 protoplasts. The Northern blot in Fig. 1B was hybridized with a probe detecting all AMV plus-strand RNAs; the probe in Fig. 1D detected plus-strand RNA 2 only. Using 35S-cDNA clones, we have shown that mutant 2-GDD-S, with two translationally silent mutations in the sequence encoding the GDD motif of P2, accumulated at WT levels in nontransgenic and P2 plants and that the mutations were retained in the progeny virus (van Rossum *et al.,* 1996). When P2 protoplasts were inoculated with RNA and CP purified from virions isolated from nontransgenic tobacco infected with WT 35S-cDNAs 1-4 or 35S-cDNAs containing the 2-GDD-S mutation, similar levels of accumulation of RNAs 1-4 were observed (Fig. 1B, lanes 2 and 3). Similarly, when nontransgenic tobacco was inoculated with T7 RNA polymerase transcripts of clones pUT17A, pUT27A, and pAL3 or transcripts containing the 2-GDD-S mutation and RNA and CP purified from virus isolated from these plants was used to inoculate P2 protoplasts, accumulation of RNAs 1–4 was observed in the protoplasts (Fig. 1B, lanes 4 and 5). However, when the P2 protoplasts were directly inoculated with CP and transcripts of pUT17A, pUT27A, and pAL3 or transcripts containing the 2-GDD-S mutation, only accumulation of RNAs 1, 3, and 4 was observed (Figs. 1B and 1D, lanes 6 and 7). Apparently, RNA 2 transcribed from pUT27A replicated at WT levels in nontransgenic and P1 protoplasts but was unable to compete with RNAs 1 and 3 for the replication machinery in P2 protoplasts under conditions where it was not required for infection. When the translationally nonsilent mutations 2-GGD and 2-VDD were introduced in the GDD motif encoded by RNA 2, the virus became noninfectious to nontransgenic plants and RNA 2 no longer coreplicated with RNAs 1 and 3 in P2 plants (van Rossum *et al.,* 1996). As expected, T7 transcripts of mutants 2-GGD and 2-VDD did not coreplicate with RNAs 1 and 3 in P2 protoplasts (Figs. 1B and 1D, lanes 8 and 9). However, no conclusion about complementation of these mutants could be drawn because the control transcript of clone PUT27A did not replicate in the presence of transgenic P2.

Cis- and *trans*-acting functions of CP

Previously, we showed that RNA 3 mutants with defective P3 and CP genes could not complement each other in infected P12 protoplasts (van der Vossen*et al.,* 1996). Here, we used nontransgenic protoplasts to investigate *trans*- and *cis*-acting functions of CP in replication of RNAs 1–3 and in encapsidation of these RNAs. Figure 2, lane 1, shows the accumulation of plus-strand RNAs 1–4 in nontransgenic protoplasts inoculated with T7 RNA polymerase transcripts of clones pUT17A, pUT27A, and pAL3; in all experiments shown in Fig. 2, CP was added to the inoculum. The infection shown in lane 1 of Fig. 2A is referred to as the "WT" infection. The accumulation of CP in the WT infected protoplasts is shown in Fig. 3, lane 1. Mutant 3-ΔP3 contains a deletion of 310 nucleotides in the P3 gene and can be distinguished from WT RNA 3 on Northern blots by its enhanced migration rate. The deletion does not interfere with the accumulation of RNAs 1 and 2 and mutant RNA 3 (Fig. 2A, lane 2) or the accumulation of CP (Fig. 3, lane 2). (It should be noted that CP of strain YSMV, expressed by 3-ΔP3, migrates slightly faster than CP of strain 425, expressed by the transcript of pAL3.) In Fig. 2B, the Northern blot of Fig. 2A was hybridized with probe X-H corresponding to the sequence that is deleted in 3-ΔP3. This probe detects WT RNA 3 (Fig. 2B, lane 1) but not RNA 3 of mutant 3-ΔP3 (Fig. 2B, lane 2). In mutant 3-ΔAUG, the initiation codon of the CP gene is changed into AAG and expression of the CP gene is abolished (van der Vossen *et al.,* 1994). Accumulation of RNAs 1–3 is strongly reduced by the

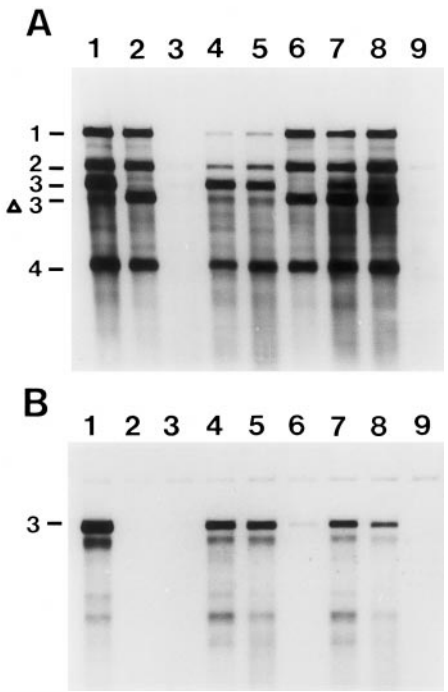

FIG. 2. Replication of RNA 3 mutants in nontransgenic protoplasts. Protoplasts were inoculated with a mixture of CP and T7 transcripts of clones pUT17A and pUT27A (lanes 9) or this mixture to which RNA 3 was added that was transcribed from pAL3 (lanes 1), 3-ΔP3 (lanes 2), 3-ΔAUG (lanes 3), 3–4P (lanes 4), 3-N199 (lanes 5), 3-ΔP3 and 3-ΔAUG (lanes 6), 3-ΔP3 and 3–4P (lanes 7), and 3-ΔP3 and 3-N199 (lanes 8). RNA extracted from the protoplasts was analyzed by Northern blot hybridization, and the blots were hybridized to a probe detecting plus-strand RNAs 1–4 (A) or to probe X-H, detecting WT RNA 3 and RNA 3 of CP mutants but not RNA 3 of mutant 3-ΔP3 (B). The position of RNAs 1–4 and RNA 3 of mutant 3-ΔP3 (Δ3) is indicated in the left margin.

mutation (Figs. 2A and 2B, lanes 3), demonstrating that CP expressed from RNA 3 is required in *trans* for the accumulation of RNAs 1 and 2. When protoplasts were infected with RNAs 1 and 2 transcripts only, plus-strand RNA accumulation was as low as in protoplasts infected with mutant 3-ΔAUG (Fig. 2A, lane 9). In mutant 3-4P, amino acids 85 and 86 of the CP are replaced by 6 nonviral amino acids, and in mutant 3-N199, the C-terminal 21 amino acids of the CP are replaced by 4 nonviral amino acids. Previously, we showed that in P12 protoplasts, mutant 3-4P accumulated at near-WT levels, whereas the accumulation of 3-N199 was strongly reduced (van der Vossen *et al.,* 1994). In nontransgenic protoplasts, RNA 3 of mutant 3-4P (Fig. 2B, lane 4) and mutant 3-N199 (Fig. 2B, lane 5) accumulated at similar levels, which were somewhat lower than the WT level (Fig. 2B, lane 1). The mutants expressed near WT levels of CP (Fig. 3, lanes 3 and 4), and these mutant CPs supported the accumulation of RNAs 1 and 2 to levels that were somewhat lower than WT (Fig. 2A, lanes 4 and 5).

To see whether CP expressed by mutant 3-ΔP3 could complement the defect in RNA accumulation of mutant 3-ΔAUG, protoplasts were infected with a mixture of the two mutants. In this mixed infection, the CP of mutant 3-ΔP3 was expressed at WT levels (result not shown), and this CP supported accumulation of RNA 1, RNA 2, and RNA 3 of mutant 3-ΔP3 to WT levels (Fig. 2A, lane 6) but virtually did not stimulate accumulation of RNA 3 of mutant 3-ΔAUG (Fig. 2B, lane 6). Also, in mixed infections of 3-ΔP3 and 3–4P or 3-ΔP3 and 3-N199, no clear stimulation of RNA 3 of the CP mutants by the WT CP of the 3-ΔP3 mutant was observed (Figs. 2A and 2B, lanes 7 and 8), although there is efficient accumulation of WT and mutant CP in these protoplasts (Fig. 3, lanes 5 and 6). These observations support the notion that CP is required in *trans* for replication of RNAs 1 and 2 and is required in *cis* for replication of RNA 3.

To analyze *cis*- and *trans*-acting functions of CP in encapsidation, nontransgenic protoplasts infected with P3 and CP mutants were homogenized at room temperature in PE buffer to degrade nonencapsidated RNA (van der Vossen *et al.,* 1994). Virus particles in the homogenate migrate as a single band in agarose gels and can be detected by Northern blot hybridization. Lanes 1–9 of Fig. 4 show the virus particles assembled in the protoplasts that were analyzed for RNA accumulation in lanes 1–9 of Fig. 2. The blot of Fig. 4 was hybridized with Fig. 4A, probe X-N, which detects RNA 3 of the WT, the 3-ΔP3 mutant, and the CP mutants; Fig. 4B, probe X-H, which detects RNA 3 of the WT and the CP mutants; and Fig. 4C, a probe detecting RNA 1; Fig. 4D, a probe detecting RNA 2. In protoplasts infected with WT RNAs (Fig. 4, lanes 1) and with mutant 3-ΔP3 (Fig. 4, lanes 2), RNAs 1–3 are encapsidated. As a control, it can be seen that RNA 3 of mutant 3-ΔP3 is detected by the X-N probe in Fig. 4A, lane 2, but not by the X-H probe in Fig. 4B, lane 2. In protoplasts infected with mutant 3-ΔAUG, there is no production of CP and virtually no accumulation of RNA; consequently, no encapsidation of RNA 1–3 is detectable (Fig. 4, lanes 3). Using P12 protoplasts, we have previously shown that CPs of mutants 3-4P and 3-N199 are defective in encapsidation of RNAs 3 and 4 (van der Vossen *et al.,* 1994). In nontransgenic protoplasts in-

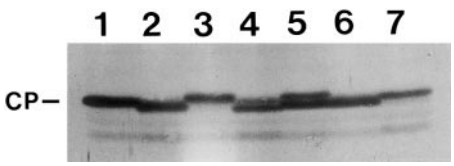

FIG. 3. Accumulation of CP in nontransgenic protoplasts infected with RNA 3 mutants. The protoplasts were inoculated with a mixture of CP and T7 transcripts of clones pUT17A and pUT27A to which RNA 3 was added that was transcribed from pAL3 (lane 1), 3-ΔP3 (lane 2), 3-4P (lane 3), 3-N199 (lane 4), 3-ΔP3 and 3–4P (lane 5), and 3-ΔP3 and 3-N199 (lane 6). CP accumulation in the protoplasts was analyzed by the Western blot technique using antiserum against AMV CP. Lane 7 was loaded with purified CP of AMV strain 425. The position of CP is indicated in the left margin.

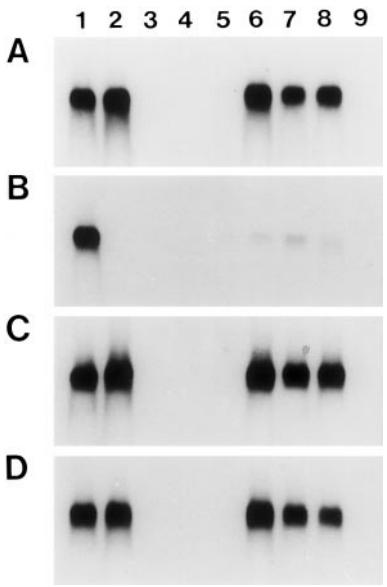

FIG. 4. Encapsidation of RNAs 1–3 in nontransgenic protoplasts infected with RNA 3 mutants. Protoplasts were inoculated with a mixture of CP and T7 transcripts of clones pUT17A and pUT27A (lanes 9) or with this mixture to which RNA 3 was added that was transcribed from pAL3 (lanes 1), 3-ΔP3 (lanes 2), 3-ΔAUG (lanes 3), 3–4P (lanes 4), 3-N199 (lanes 5), 3-ΔP3 and 3-ΔAUG (lanes 6), 3-ΔP3 and 3–4P (lanes 7), and 3-ΔP3 and N-199 (lanes 8). The protoplasts were homogenized and incubated at room temperature to degrade nonencapsidated RNA. Virus particles in the homogenates were electrophoresed in agarose gels and visualized by Northern blot hybridization using probe X-N, which detects all RNA 3 molecules used for inoculation (A), probe X-H, which detects WT RNA 3 and RNA 3 of the CP mutants, but not RNA 3 of mutant 3-ΔP3 (B), a probe detecting RNA 1 (C), or a probe detecting RNA 2 (D).

fected with these mutants, there is substantial accumulation of RNAs 1–4 (Fig. 2, lanes 4 and 5) and CP (Fig. 3, lanes 3 and 4), but none of the RNAs is encapsidated (Fig. 4, lanes 4 and 5). Apparently, CP of these mutants is also defective in encapsidation of RNAs 1 and 2. When protoplasts are infected with a mixture of mutant 3-ΔP3 and 3–4P (Fig. 4, lanes 7) or 3-ΔP3 and 3-N199 (Fig. 4, lanes 8), RNAs 1 and 2 are encapsidated (Figs. 4C and 4D, lanes 7 and 8) with an efficiency similar to that in protoplasts infected with 3-ΔP3 only (Figs. 4C and 4D, lanes 2). However, RNA 3 of the CP mutants is virtually not encapsidated by the WT CP expressed by mutant 3-ΔP3 (Fig. 4B, lanes 7 and 8). From this observation, it can be concluded that the signals present in lanes 7 and 8 of Fig. 4A reflect the encapsidation of RNA 3 of mutant 3-ΔP3 only. Apparently, CP expressed by RNA 3 of mutant 3-ΔP3 is able to encapsidate RNAs 1 and 2 in *trans* but cannot encapsidate in *trans* RNA 3 of the CP mutants. It is concluded that encapsidation of RNA 3 requires the CP in *cis*. To complete the analysis, encapsidation of viral RNAs was also assayed for protoplasts infected with a mixture of mutants 3-ΔP3 and 3-ΔAUG (Fig. 4, lanes 6) and with RNA 1 and RNA 2 transcripts only (Fig. 4, lanes 9).

Early and late functions of CP in P12 protoplasts

Previously, we showed that CP is not required to initiate infection of P12 protoplasts with RNA 3, but attempts to study the role of CP in replication of RNAs 1 and 2 in P12 protoplasts were hampered by minor contaminations of RNA 3 in the RNA 1 and 2 preparations (Taschner *et al.,* 1991). The availability of infectious transcripts of RNAs 1 and 2 permitted a reinvestigation of this question. P12 protoplasts were inoculated with T7 RNA polymerase transcripts of pUT17A, pUT27A, and pAL3, either alone or in various combinations, and inoculation was done plus or minus CP in the inoculum. The Northern blots of Fig. 5 show the accumulation in these P12 protoplasts of plus-strand RNAs 1 and 2 (Fig. 5A), plus-strand RNAs 3 and 4 (Fig. 5B), minus-strand RNAs 1 and 2 (Fig. 5C), and minus-strand RNA 3 (Fig. 5D). Lanes 1 of Fig. 5 show an analysis of mock-inoculated protoplasts. In lanes 2 and 3 of Fig. 5, the protoplasts were inoculated with the RNA 1 transcript minus or plus CP, respectively. The relatively low signal seen in Fig. 5A, lane 2, may represent inoculum RNA because it is not paralleled by the synthesis of minus-strand RNA 1 in lane 2 of Fig. 5C. However, after the addition of CP to the inoculum, the synthesis of minus-strand RNA 1 becomes detectable (Fig. 5C, lane 3). Using virus particles as

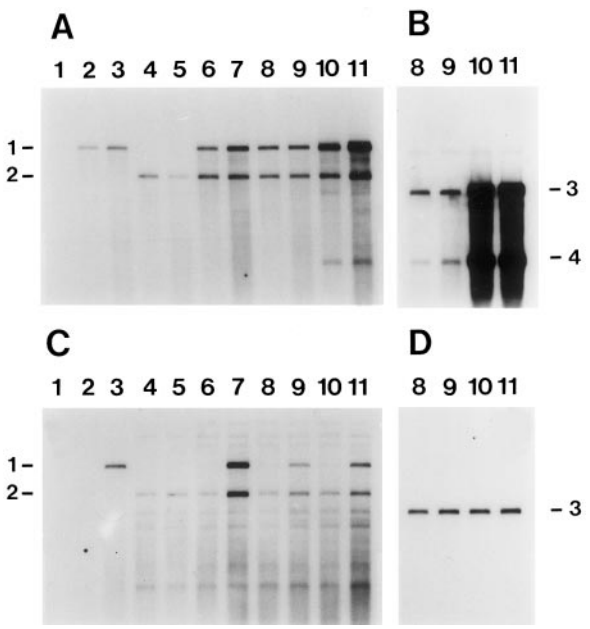

FIG. 5. Accumulation of plus- and minus-strand RNAs 1–3 in P12 protoplasts. Protoplasts were mock inoculated (lanes 1) or inoculated with T7 transcripts of clones pUT17A (lanes 2 and 3), pUT27A (lanes 4 and 5), pUT17A and pUT27A (lanes 6 and 7), pUT17A, pUT27A, and 3-ΔAUG (lanes 8 and 9), and pUT17A, pUT27A, and pAL3 (lanes 10 and 11). Inoculation was done with (lanes 3, 5, 7, 9, and 11) or without (lanes 2, 4, 6, 8, and 10) CP in the inoculum. RNA extracted from the protoplasts was analyzed by Northern blot hybridization using probes detecting plus-strand RNAs 1 and 2 (A), plus-strand RNAs 3 and 4 (B), minus-strand RNAs 1 and 2 (C), or minus-strand RNA 3 (D). The positions of RNAs 1–4 are indicated in the margins.

inoculum, we have previously shown that RNA 1 is able to replicate in P12 protoplasts in the absence of RNA 2, probably by using the transgenic P2 that is present in excess over P1 in these protoplasts (van Rossum *et al.,* 1996). Apparently, the initiation of RNA 1 replication in P12 protoplasts requires CP in the inoculum. In lanes 4 and 5 of Fig. 5, the protoplasts were inoculated with the RNA 2 transcript minus or plus CP, respectively. The weak signal in lane 4 of Fig. 5C is probably artifactual. Control experiments have shown that the plus-strand RNA probe used to detect minus-strand RNA 2 weakly cross-hybridized with the plus-strand RNA 2 transcript used to inoculate the protoplasts (data not shown). No increase in the signal in lane 4 of Fig. 5C, or the signal obtained with the probe detecting plus-strand RNA 2 (lane 4 of Fig. 5A), was observed after addition of CP to the inoculum (Figs. 5A and 5C, lanes 5). This is in agreement with our previous observation that replication of RNA 2 in P12 protoplasts is dependent on the presence of RNA 1 in the inoculum (van Rossum *et al.,* 1996). When P12 protoplasts were inoculated with a mixture of RNA 1 and 2 transcripts without CP, no accumulation of minus-strand RNAs was detectable (Fig. 5C, lane 6). However, when CP was added to the inoculum, accumulation of minus-strand RNAs 1 and 2 was observed (Fig. 5C, lane 7). This minus-strand RNA synthesis was accompanied by only a minor increase in the level of plus-strand RNAs in the protoplasts (compare lanes 6 and 7 of Fig. 5A). Probably, the plus-strand RNAs 1 and 2 detectable in lane 6 of Fig. 5A represent inoculum RNAs, and after the addition of CP, these inoculum RNAs served as templates for synthesis of the minus-strand RNAs seen in lane 7 of Fig. 5C. The addition of RNA 3 mutant 3-ΔAUG (Fig. 5, lanes 8 and 9) or WT RNA 3 (Fig. 5, lanes 10 and 11) to inocula containing RNAs 1 and 2 minus or plus CP did not change the pattern of minus-strand RNA synthesis (compare lanes 6 and 7, 8 and 9, and 10 and 11 of Fig. 5C). In each case, CP was required in the inoculum to permit synthesis of minus-strand RNAs 1 and 2. The addition of mutant 3-ΔAUG did not affect accumulation of plus-strand RNAs 1 and 2 (Fig. 5A, lanes 8 and 9). However, the addition of WT RNA 3 did result in an increased accumulation of plus-strand RNAs 1 and 2 (Fig. 5A, lanes 10 and 11). When P12 protoplasts were inoculated with RNAs 1–3-ΔAUG, minus-strand RNA 3 synthesis was detectable regardless of the presence of CP in the inoculum (Fig. 5D, lanes 8 and 9). These minus-strand RNAs served as templates for the accumulation of relatively low levels of plus-strand RNAs 3 and 4 (Fig. 5B, lanes 8 and 9). When mutant 3-ΔAUG in the inoculum was replaced by WT RNA 3, no increase in minus-strand RNA 3 accumulation was detectable (Fig. 5D, lanes 10 and 11), but a strong increase in the accumulation of plus-strand RNAs 3 and 4 was observed (Fig. 5B, lanes 10 and 11). The data on plus- and minus-strand synthesis of mutant 3-ΔAUG and WT RNA 3 in P12 protoplasts are in agreement with results published previously (van der Vossen *et al.,* 1994).

DISCUSSION

T7 transcripts corresponding to AMV RNAs 1 and 2 with the U residue at position 3 changed into an A residue (RNAs 1-U3A and 2-U3A) replicated at WT levels in nontransgenic plants and protoplasts. In P1 or P2 plants and protoplasts, however, these transcripts only replicated at WT levels when their encoded gene products were required for replication of RNA 3. Previously, we have shown that the 5′ sequence GUAUU of RNA 3 contains a *cis*-acting element that is involved in directing the viral RdRp to initiation at the 3′ end of minus-strand RNA 3 (van Rossum *et al.,* 1997). When this element was deleted or replaced by the sequence GCCCC or GUCCC, the RdRp selected a C residue in the minus-strand, corresponding to G residue 80 in plus-strand RNA 3, to initiate plus-strand RNA 3 synthesis. When the element was replaced by the 5′ sequence GUUUU, corresponding to the 5′ sequence of RNAs 1 and 2, plus-strand RNA 3 synthesis started at the 3′ end of the minus-strand RNA 3 template, but the ability of this GUUUU mutant to compete with WT RNA 3 was not tested (van Rossum *et al.,* 1997). The work reported here indicated that the U residue at position 3 of RNAs 1 and 2 also is part of a *cis*-acting element involved in RNA replication. The U3A mutation does not destroy this *cis*-acting function but reduced the ability of the RNAs to compete with other viral RNAs for the replication machinery (Fig. 1A, lane 3; Fig. 1B, lane 6). When RNA 2-U3A was passaged in nontransgenic plants, the ability of the RNA to coreplicate with RNAs 1 and 3 in P2 protoplasts was restored (Fig. 1B, lane 4). We have not yet investigated whether the U3A mutation reverted to WT during passage of the virus. Also, efficient capping of the RNA *in vivo* might have increased the fitness of the passaged RNA over less efficiently capped transcripts made *in vitro*.

Although coreplication of RNA 1-U3A with RNAs 2 and 3 in P1 protoplasts was less efficient than coreplication of WT RNA 1, the level was sufficient to demonstrate the role of the P1 reading frame in RNA 1-U3A for this coreplication (Fig. 1A, lanes 4 and 5). A reduced translatability of RNA 1 mutants 1-fsP and 1-fsN probably is not the reason for the inability of these mutants to use the transgenic P1 for replication in *trans*. Ninety percent of the reading frame of the 126-kDa P1 protein is still intact in mutant 1-fsN. Also for RNA 3, frameshift mutations in the P3 gene that abolished translatability of the RNA did not affect its replication in *trans* by the transgenic RdRp in P12 protoplasts (van der Kuyl *et al.,* 1991a). By a study of the accumulation of 18 P1 and P2 mutants in P1 and P2 plants, we previously concluded that RNAs 1 and 2 cannot use the transgenic P1 and P2 in trans, respectively, and require their encoded P1 and P2 in *cis*

for accumulation. The analysis of mutants 1-fsP and 1-fsN reported here supports the notion that P1 and P2 have *cis*-acting functions in RNA replication rather than in cell-to-cell movement. These *cis*-acting functions may be used by the virus to eliminate nonviable mutants from the virus population or to ensure that RdRp and viral template RNA are targeted together to cellular membranes where replication complexes are established (van Rossum *et al.,* 1996).

When the requirement of CP for the initiation of AMV infection was discovered, several hypotheses were put forward to explain a possible role of CP in either translation or replication of the inoculum RNAs (Bol *et al.,* 1971). The finding of specific binding sites for CP at the 3′ end of AMV RNAs supported the notion that binding of CP to these sites was required for the initiation of minus-strand RNA synthesis by the viral RdRp (Houwing and Jaspars, 1978). This hypothesis was abandoned when it became clear that a block in the expression of the CP gene in RNA 3 did not affect minus-strand RNA 3 synthesis in P12 protoplasts (van der Kuyl *et al.,* 1991a; van der Vossen *et al.,* 1994) and that plus-strand AMV template RNA was efficiently transcribed *in vitro* into minus-strand RNA by a CP-free RdRp isolated from healthy P12 plants (de Graaff and Jaspars, 1995). When cowpea protoplasts were inoculated with purified virus particles containing AMV RNAs 1 and 2, a strong reduction in the accumulation of plus-strand RNAs 1 and 2 was observed compared with an infection with the complete genome (Nassuth and Bol, 1983). A mutational analysis of the P3 and CP genes confirmed that the CP gene was the RNA 3-encoded function required for plus-strand RNA 3 accumulation in P12 protoplasts (van der Kuyl *et al.,* 1991a). In the present work, the availability of infectious transcripts of RNAs 1 and 2 permitted the demonstration that expression of the CP gene in RNA 3 is required not only for the accumulation of plus-strand RNA 3 but also for the accumulation of plus-strand RNAs 1 and 2 (Fig. 2A, lane 3). Mutants 3-4P and 3-N199 express mutant CPs that support near-WT levels of plus-strand RNA accumulation but do not encapsidate the RNAs (Figs. 2 and 4, lanes 4 and 5). This indicates that the low level of accumulation of plus-strand RNAs 1–4 by mutant 3-ΔAUG reflects a low level of synthesis rather that a high level of degradation, due to a possible instability of the RNAs in the absence of encapsidation.

CP expressed by mutant 3-ΔP3 was able to upregulate synthesis of plus-strand RNAs 1 and 2 and to encapsidate these RNAs. However, this CP did not upregulate plus-strand RNA 3 synthesis of mutant 3-ΔAUG, nor did it encapsidate plus-strand RNA 3 of mutants 3-4P and 3-N199. Apparently, CP expressed by WT RNA 3 acts in *trans* in plus-strand synthesis and encapsidation of RNAs 1 and 2 and acts in *cis* in plus-strand synthesis and encapsidation of RNA 3. This indicates that in the infected cell, RNA 3 replication, RNA 4 synthesis, CP synthesis, and encapsidation of RNA 3 are tightly coupled.

In various models to explain the role of CP in AMV replication, it has been proposed that the early function of CP in the inoculum is linked to its role in plus-strand RNA synthesis (de Graaff and Jaspars, 1995) or that different functions of CP are involved in genome activation and plus-strand RNA synthesis (Neeleman *et al.,* 1993). To shed light on these two possibilities, we analyzed the replication of RNAs 1 and 2 in P12 protoplasts. In P12 protoplasts, RNA 3 accumulation is independent of CP in the inoculum and starts immediately after inoculation, whereas accumulation of RNAs 1 and 2 becomes detectable from 6 h after inoculation, as in nontransgenic protoplasts. It has been proposed that the *cis*-acting functions of P1 and P2 preclude replication of RNAs 1 and 2 by the transgenic RdRp in *trans* (van Rossum *et al.,* 1996). The results presented in Fig. 5 demonstrate that CP is required in the inoculum to permit replication of RNAs 1 and 2 in P12 protoplasts. Apparently, CP expressed from the CP gene in RNA 3 is unable to fulfill the early function of CP required for the initiation of replication of RNAs 1 and 2. This indicates that also in nontransgenic cells, the role of the coat protein in the inoculum is not solely to permit expression of the CP gene in RNA 3. This is in agreement with our earlier observation that the early function of CP requires the presence of CP in a narrow time window after inoculation (Neeleman *et al.,* 1993). CP in the inoculum appeared to be required for minus-strand RNA 1 and 2 synthesis in P12 protoplasts (Fig. 5C, lanes 6–11). Previously, we proposed that CP in the inoculum is required to protect the 3′ termini of the genomic RNAs from exonucleolytic degradation during translation of RNAs 1 and 2 into an RdRp activity. Possibly, such a protective activity permits the synthesis of minus-strand RNAs 1 and 2 seen in lanes 7, 9, and 11 of Fig. 5C.

When the expression of the CP gene in RNA 3 is blocked in mutant 3-ΔAUG, a low level of plus-strand RNA synthesis is observed in P12 protoplasts, as demonstrated by the detection of RNA 4 in lanes 8 and 9 of Fig. 5B. Because the probes used in Fig. 5 detect plus- and minus-strand RNAs with similar sensitivity, a comparison of lanes 8 and 9 of Figs. 5B and 5D indicates that in the absence of expression of the CP gene in RNA 3, similar amounts of plus- and minus-strand RNA 3 are synthesized. However, when expression of the CP gene is permitted, plus-strand RNA 3 synthesis is strongly stimulated (Fig. 5B, lanes 10 and 11; van der Vossen *et al.,* 1994). In summary, we presented three observations that indicate that AMV CP fulfills different functions in genome activation and RNA replication: (1) CP in the inoculum is required for minus-strand RNA synthesis, whereas CP expressed from inoculum RNA 3 is required for plus-strand RNA synthesis; (2) CP expressed from RNA 3 cannot fulfill the function of CP in the inoculum;

and (3) in nontransgenic plants or protoplasts, CP in the inoculum functions in *trans* in the initiation of RNA 3 replication, whereas it is required in *cis* for plus-strand RNA 3 synthesis.

MATERIALS AND METHODS

Construction of infectious clones

Clones pUT17A and pUT27A contain full-length cDNAs of AMV RNAs 1 and 2 (strain 425), respectively, fused precisely to the T7 RNA polymerase promoter. In these clones, the 5′ sequence GUUUUU of RNAs 1 and 2 was modified into GUAUUU. For the construction of pUT17A, RNA 1 was transcribed with reverse transcriptase into single-stranded cDNA using a primer complementary to nucleotides 269–288 of RNA 1. To this cDNA, the following oligonucleotide was annealed: 5′ CAGG**AAGCTT**TAATACGAGTCACTATAGTATTTATCTTACACACGCTTGTG 3′. This primer contains a *Hin*dIII restriction site (bold letters), a T7 promoter sequence, and a sequence corresponding to the 5′ 24 nucleotides of RNA 1 (underlined) and was used to prime second-strand cDNA using *Taq* polymerase (GIBCO BRL). The cDNA was digested with *Hin*dIII at the 5′ end and with *Pst*I at position 204 of RNA 1. The fragment containing the 5′ sequence of RNA 1 (nucleotides 1–204) was fused to a *Pst*I–*Sma*I fragment containing the remaining sequence of RNA 1 (nucleotides 205–3644), corresponding to the infectious 35S-cDNA 1 clone pCa17T described by Neeleman *et al.* (1993). The *Hin*dIII–*Sma*I fragment containing the T7 promoter–cDNA 1 construct was inserted into *Hin*dIII–*Sma*I digested pUC9. In a similar way, pUT27A was constructed using a primer for first-strand cDNA synthesis complementary to nucleotides 789–806 of RNA 2 and performing second-strand synthesis with the primer: 5′ CAGG**AAGCTT**TAATACGAGCTACTATAGTATTTATCTTTTCGCG 3′. This primer contains a *Hin*dIII restriction site (bold letters), a T7 promoter sequence, and a sequence corresponding to the 5′ 24 nucleotides of RNA 2. The fragment was digested with *Hin*dIII at the 5′ end and with *Xho*I at position 262 of RNA 2. The fragment containing the 5′ end of RNA 2 (nucleotides 1–262) was fused to the *Xho*I–*Pst*I fragment containing the remaining sequence of RNA 2 (nucleotides 263–2142), corresponding to the infectious 35S-cDNA 2 clone pCa27T (Neeleman *et al.,* 1993). The HindIII–*Pst*I fragment containing the T7 promoter/cDNA 2 construct was inserted into *Hin*dIII–*Pst*I-digested pUC9.

Clone pAL3 described by Neeleman *et al.* (1991) was transcribed *in vitro* with T7 RNA polymerase into infectious RNA 3 transcripts of AMV strain 425.

Mutant AMV cDNA clones

Mutant 3-ΔP3 was made by deleting the sequence in the P3 gene of clone pYSMV3 from the *Xho*I site at position 415 to the *Hin*dIII site at position 725 of RNA 3 of AMV strain YSMV (Neeleman *et al.,* 1991). Mutations in the CP gene of RNA 3 of strain 425 were engineered in clone pAL3 and have been described before (van der Vossen *et al.,* 1994). In mutant 3-ΔAUG, the initiation codon of the CP gene is changed into AAG. In mutant 3–4P, amino acids Arg and Ile at position 85/86 of the CP are replaced by the sequence ProAlaGlyLeuGlnVal. Mutant 3-N199 contains a frameshift in the CP reading frame resulting in a replacement of the C-terminal 21 amino acids by 4 nonviral amino acids.

Mutant 1-fsP was made by digesting pUT17A with *Pst*I at position 204 of RNA 1 and religation after the ends had been made blunt with T4 DNA polymerase. This mutant contains a frameshift early in the P1 reading frame (deletion of nucleotides 205–208). The following mutations in RNAs 1 and 2 have been described previously (van Rossum *et al.,* 1996) and were transferred from the mutated 35S-cDNA clones pCa17T and pCa27T into the T7-cDNA clones pUT17A and pUT27A, respectively, by exchanging appropriate restriction fragments. Mutant 1-fsN contains an insertion of 4 nucleotides between nucleotides 3331 and 3332 of RNA 1, resulting in a frameshift near the 3′ end of the P1 reading frame. Mutant 2-GDD-S contains two translationally silent mutations in the GDD motif encoded by the P2 gene in RNA 2. In mutants 2-GGD and 2-VDD, the amino acid sequence GDD of the GDD motif in P2 is changed into GGD and VDD, respectively.

Transcription of AMV cDNAs *in vitro*

Plasmids containing WT or mutant AMV cDNA 1 were linearized with *Sma*I, whereas plasmids containing WT or mutant cDNAs 2 and 3 were linearized with *Pst*I. After *Pst*I digestion, the ends were made blunt with T4 DNA polymerase. *In vitro* transcription with T7 RNA polymerase was done with 2 μg of template DNA in a reaction volume of 100 μl as described previously (van der Kuyl *et al.,* 1991b). Transcription of WT and mutant RNAs 1 and 2 was done in the presence of 0.2 mM of the cap analog G(5′)ppp(5′)G (New England Biolabs). At the 5′ end, the *in vitro* synthesized transcripts will not contain nonviral nucleotides. At the 3′ end, the RNA 1 transcripts will contain two nonviral C residues.

Inoculation of protoplasts

Protoplasts were prepared from nontransgenic Samsun NN tobacco plants and transgenic P1, P2, and P12 plants (Taschner *et al.,* 1991; van Dun *et al.,* 1988), and samples of 2.5×10^5 protoplasts were inoculated with transcription mixtures containing RNAs 1, 2, and/or 3 (10 μl per transcription mixture). When indicated, AMV CP (strain 425) was added to the inoculum to a level of 40 CP molecules per RNA molecule in the inoculum. Isolation and inoculation of protoplasts were done as described

previously (Loesch-Fries *et al.,* 1985; van Dun *et al.,* 1988). CP was prepared as described by Neeleman *et al.* (1993). Protoplasts were incubated for 18 h at 25° under continuous illumination.

Analysis of inoculated protoplasts

Total RNA was extracted from protoplasts using TRIzol Reagent (GIBCO BRL), denatured by glyoxal/DMSO treatment, and analyzed by Northern blot hybridization as described previously (van der Vossen *et al.,* 1996). Viral RNAs were visualized on blots using DIG-labeled riboprobes (Boehringer-Mannheim). Riboprobes for strand-specific detection of plus-strand and minus-strand RNAs were prepared by transcription with T7 RNA polymerase of AMV cDNAs 1–3 cloned behind the T7 promoter in antisense and sense direction, respectively. Linearization of the cDNA constructs was done with restriction enzymes that created ends with a 5′ overhang and digested the DNA in the polylinker downstream of the cDNA. Sense cDNAs 1–3 were linearized with *Eco*RI, *Bgl*II, and *Eco*RI, respectively, and antisense cDNAs 1–3 with *Bam*HI, *Bam*HI, and *Hin*dIII, respectively. Probes X-H and X-N were prepared by cloning the *Xho*I–*Hin*dIII fragment (nucleotides 415–725 of RNA 3) and the *Xho*I–*Nde*I fragment (nucleotides 415–1298 of RNA 3) from clone pYSMV3 (Neeleman *et al.,* 1991) into pUC21 in antisense orientation behind the T7 promoter. After linearization, the inserts were transcribed *in vitro* with T7 RNA polymerase. Probe X-H will detect WT RNA 3 and all RNA 3 mutants except mutant 3-ΔP3. Probe X-N will detect WT RNA 3 and all mutant RNA 3 molecules. Moreover, probes X-H and X-N will not detect RNA 4. Labeling and detection of the riboprobes were carried out according to the manufacturer's instructions.

Encapsidation of AMV RNAs into virus particles was analyzed by sedimenting 0.5×10^5 protoplasts by centrifugation. The protoplasts were resuspended in 100 μl of PE buffer (0.01 M NaH_2PO_4, 1 mM EDTA, pH 7.0), homogenized, and incubated for 30 min at room temperature to degrade nonencapsidated RNAs. After centrifugation, 20 μl of the supernatant was electrophoresed in a 0.8% agarose gel, and the virions were visualized by Northern blot hybridization (van der Vossen *et al.,* 1996).

For protein analysis, 0.5×10^5 protoplasts were pelleted by centrifugation and resuspended in 50 μl of Laemmli loading buffer (Laemmli, 1970). CP accumulation was monitored by Western blot analysis (Towbin *et al.,* 1979) using antiserum against AMV CP.

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